pH responsive MMT/Acrylamide Super Composite Hydrogel: Characterization, Anticancer Drug Reservoir and Controlled Release Property

Composite hydrogel:Drug Delivery

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Abstract

We report here, the preparation and characterization of polyacrylamide/Na^+-MMT (Montmorillonite) composite hydrogels and its application as anticancer drug carrier reservoir. The X-ray diffraction patterns, thermal and spectroscopic analyses indicate that the acrylamide and MMT has been successfully conglomerated to form composite gels. The degree of swelling was higher in composite hydrogels as compared to pristine hydrogel; and the hydrophilic MMT improved the water absorbing properties of composite hydrogels. The mode of water transport in the composite hydrogels was partially through a swollen polymeric matrix and water filling in clay layers of the composite hydrogels. *In vitro* drug release study demonstrated the controlled release pattern. *In vitro* cytotoxicity of drug loaded composite hydrogel was evaluated in MCF-7 and Hela cells by MTT assay. *In vivo* pharmacokinetics, biodistribution, toxicity biomarkers and total serum protein analysis were also investigated after single oral administration of drug in rats.

Keywords

Composite Hydrogel; Biomarkers; Cytotoxicity; Pharmacokinetics; Na^+-MMT

Introduction

Hydrogels are networks of hydrophilic cross-linked polymers that swell in the presence of water or physiological fluids. During the last two decades, polymer/clay composite hydrogels have been the topic of extensive research in the field of drug delivery and tissue engineering. Addition of clay in hydrogels can improve physicochemical properties (such as mechanical properties, thermal stability, swelling capability and bactericidal activity) of the polymeric materials and also provide controlled release of the drug in comparison to the pristine polymers (Drury and Mooney, 2003; Xiang et al., 2006; Yi and Zhang, 2007; Kevadiya et al., 2011). The advantages of clay based hydrogels compared to pristine one are reproducibility, freedom from biological contamination, null toxicity and ability to tune properties by modifications. The "smart hydrogels" have been a subject of special curiosity due to their capability to respond, with pronounced property changes to external stimuli (Li et al., 2009; Galperin et al., 2010). The incorporation of layered silicates such as montmorillonite (MMT) into a polymer hydrogel could increase the water absorption capacity, depending on dispersion level of the clay particles. Further more, ''flexibility and potency" caused by the disposition of clay particles inside the polymer matrix is another important feature that may directly affect the loading of solutes and their release process (Liang et al., 2000; Aalaie et al., 2008; Wang et al., 2009).

5-fluorouracil (5-FU) is an effective chemotherapy option available for the treatment for colorectal cancer, stomach cancer, breast cancer, brain tumor, liver cancer, pancreatic cancer and lung cancer (Arıca et al., 2002; Qian et al., 2002; Elias et al., 2004; Lesniak and

Brem, 2004; Gupta and Aggarwal, 2007; Kalantarian et al., 2010; Wang et al., 2010; Jungkyun et al., 2011; Shenoy et al., 2012). It is a pyrimidine analog that inhibits the biosynthesis of deoxyribonucleotides for DNA replication by inhibiting thymidylate synthase activity, leading to thymidine depletion, incorporation of deoxyuridinetriphosphate into DNA and cell death (Longley et al., 2003; Noordhuis et al., 2004). An additional mechanism of cytotoxicity is the incorporation of uridine triphosphate into RNA, which interrupts RNA anabolism and processing (Nicolay et al., 2009). However, 5-FU has limitations such as short biological half-life due to fast degradation, partial and irregular oral absorption due to metabolism by dihydropyrimidine dehydrogenase (Diasio and Lu, 1994; Gamelin et al., 1996; Aranda et al., 1998; Schilsky et al., 1998), toxic side effects on bone marrow and non-selective action against healthy cells and alteration of the gastrointestinal microflora (Li et al., 2008; Stringer et al., 2009 a,b). For successful cancer treatment, overcoming the toxic side effects on bone marrow or gastrointestinal damage is required which might be achieved by controlled release of the drug by loading of drug in the composite hydrogel systems. Recently, various inorganic hybrid composites have emerged as an imperative class of drug delivery systems in biomaterial field. Among them, layered silicate materials e.g. montmorillonite (MMT), have attracted a great deal of attention due to its capability to release drugs in a controlled manner (Dong and Feng, 2005; Kevadiya et al., 2010), mucoadhesiveness and potent detoxification, eventually leading to high efficacy of drug. It also has the capability to adsorb dietary toxins, microbial toxins associated with gastrointestinal turbulences, hydrogen ions in acidosis, and metabolic toxins such as steroidal metabolites associated with pregnancy (Dong and Feng, 2005). Na^+-clay has also been proved to be non-toxic by clinically, biochemical and tissue morphological studies in rat models (Feng et al., 2009).

Herein, we report the preparation and characterization of layered silicate composite hydrogels based on a polyacrylamide (AM)-Montmorillonite (MMT) with controllable strength and exceptionally interconnected porous structure and their potential applications in drug delivery. The composite hydrogels were evaluated for anticancer drug loading, *in vitro* release characteristics, drug release kinetics and swelling kinetics of composite hydrogel. *In vivo* pharmacokinetics, biodistribution, toxicity biomarkers and total serum protein analysis were investigated.

Materials and Methods

Starting Materials and Reagents

Acrylamide and *N-N-N'-N'-Tetramethylenediamine (TEMED)* were purchased from Spectrochem Pvt. Ltd, India. *N, N'-Methylene bis-acrylamide (bis-acrylamide)*, sodium dodecyl sulfate (SDS), coomassie brilliant blue were from S. D. Fine-Chem Limited, India and Ammonium persulfate (APS), glycine were purchased from Qualigens Fine Chemicals, India. Cellulose acetate dialysis tube (Cutoff molecular weight at -7000) and 5-Fluorouracil (5-FU) were purchased from Sigma–Aldrich, USA. Sodium MMT (Closite Na^+) was gifted by Southern Clay Products Incorporation, USA. Nutrient broth, MRS broth and agar-agar were procured from Himedia Laboratory, Mumbai, India. Lactobacillus plantarum (MTCC 2621), Lactobacillus rhamnosus (MTCC 1408) were obtained from MTCC, IMTECH, Chandigarh, India and Lactobacillus casei (NCDC 019) were obtained from NDRI, Karnal, India. All biochemical analysis kits used for analysis of serum biochemical parameters were purchased from Span diagnostics Ltd; Surat, India. All the other reagents were of HPLC grade and were used as received. Millipore water was processed by Milli-Q plus System (Millipore Corporation, USA).

Preparation of Layered Composite Hydrogels

The layered composite hydrogels were prepared by free radical polymerization method. A stock solution of acrylamide was prepared by mixing 29% (w/v) of acrylamide and 1% (w/v) of N, N'-methylene bis-acrylamide in warm milli-Q water. MMT (5 g) was suspended in 200 ml of Milli-Q water for 24 h, followed by 1 h sonication, to which 100 ml acrylamide mix (30%) was added drop wise with a peristaltic pump. The AM: MMT ratio (6:1%, w/w) was optimized to obtain stable composite hydrogels having maximum amount of MMT, maximum swelling capability and controlled drug release profiles. The suspension was stirred (800 rpm) at room temperature (RT) for 12 h. APS (10%) and TEMED were added to initiate the reaction. This solution was allowed to stand at RT in a sterile atmosphere for 30 min to form a gel. After composite hydrogel was formed, it was washed extensively with distilled water to remove any unreacted reagent. Poly acrylamide hydrogels (AM) were prepared in the same way without MMT. Swollen hydrogels were then rapidly frozen at -80°C and freeze-dried (Virtis Bench-Top-K, Virtis Co., Gardiner, USA).

Swelling Studies

The pH-dependent swelling studies were conducted using freeze dried AM hydrogels and AM-MMT composite hydrogels. Swelling studies of AM hydrogels and AM-MMT composite hydrogels were carried out in two aqueous media: simulated gastric fluid (SGF, pH 1.2) and phosphate buffer saline (PBS, pH 7.4). Accurately weighed amounts of hydrogels (ranging from 0.5 to 0.6 g) were immersed in 100 ml of swelling medium solution and at predetermined time intervals, swollen hydrogels were removed, excess water from surface was blotted gently with paper and their weight was determined (Shimadzu, AUW220D). The dynamic weight change of the hydrogels with respect to time was calculated according to the formula:

$$Weight\ change(\%)=(Ws–Wi)/Wi\times100 \quad (1)$$

Where *Ws* is the weight of the hydrogels in the swollen state and *Wi* is the initial weight of the hydrogels.

In order to investigate the diffusion kinetics model for swelling of hydrogels, the initial 12 h swelling data were fitted to the exponential heuristic equation for $Wt/W\infty \leq 0.6$ (Xiang et al., 2006).

$$Wt/W\infty=kt^n \quad (2)$$

Where *Wt* is the weight of the hydrogel at a given time during swelling, $W\infty$ is the weight of the equilibrium swollen hydrogel at an infinitely long time, '*t*' is the swelling time, '*k*' is the characteristic constant of hydrogel, and '*n*' is characteristic exponent, which relates to the transport mode of the penetrate. Values of *n* between 0.5 and 1.0 indicate anomalous transport kinetics, and '*n*' approximately 0.5 indicates the Fickian diffusion law. The values of *n* less than 0.5 may be due to water diffusion partially through a swollen matrix and water filled in clay layers of the composite hydrogels.

Characterization

X-ray diffraction (XRD) analysis was carried out on Phillips powder diffractometer X' Pert MPD using PW3123/00 curved Ni-filtered Cu Ka radiation with a scanning of 0.25°/min 2θ range of 2–70°. Fourier transform infrared spectra (FT-IR) were recorded on Perkin–Elmer, GX-FTIR as KBr pellet over the range 4000–400 cm^{-1}. Bruker Advance II–500 spectrometer equipped with a magic angle spin probe was used for the solid state ^{13}C Solid MAS NMR study of the materials at room temperature. The samples were spinned at 8 kHz and the spectra were presented from an average of 4000 scans. Thermo gravimetric analysis (TGA) was carried out within 30–800°C at the heating rate of 10°C/min under nitrogen flow (20 ml/min) using TGA/SDTA 851e, Mettler-Toledo, Switzerland. The morphology of composite hydrogels and composite treated gastrointestinal microflora was observed in scanning electron microscope (SEM), LEO-1430VP, UK. The UV–visible absorbance of drug solutions were measured at λmax = 264 nm using UV–visible spectrophotometer UV2550 (Shimadzu, Japan), equipped with a quartz cell having a path length of 1 cm.

In Vitro Drug Studies

In vitro release of drug was carried out in Julabo shaking water bath (SW23) using buffer solutions of pH 1.2 (simulated gastric fluid) and pH 7.4 (simulated intestinal fluid) as release medium. In brief, precise amounts of pristine 5-FU was dispersed in 5 ml release medium placed into an activated cellulose dialysis tube and immersed in 150 ml release medium. While, freeze dried 5-FU-AM and 5-FU/AM-MMT composite hydrogels were openly immersed in 150 ml release medium. The temperature was maintained at 37 ± 0.5°C with the shaking frequency at 120 rpm. 1 mL aliquot was withdrawn at regular time interval and the same volume was restored with fresh medium. Samples were analyzed by HPLC. The release behavior of the drug from the hydrogels was fitted in elovich equation and parabolic diffusion kinetic models (Joshi et al., 2010).

Cell cultures

HeLa (Human cervical cancer cell line) and MCF-7 (Human breast cancer cell line) were obtained from National Repository of Animal Cell Culture, National Centre for Cell Sciences (NCCS), Pune, India. MCF-7 cells were cultured in 25 cm^2 tissue culture flasks maintained at 37°C in a humidified environment of 5% CO2. HeLa cell line was grown in DMEM with 10% FBS and 1% penicillin, streptomycin and amphotericin B (PSA) antibiotic mixture whereas. MCF-7 was grown in RPMI 1640 with 10% FBS and 1% penicillin, streptomycin and amphotericin B (PSA) antibiotic mixture supplemented with 1.0 mM Sodium Pyruvate and 0.01 mg/ml insulin.

In Vitro Cytotoxicity Assay

The viability of cancer cells treated with 5-FU, AM, MMT, 5-FU-AM and 5-FU/AM-MMT composite hydrogel was evaluated by the MTT assay. 150 μl of HeLa and MCF-7 cells were seeded in 96-well plates

(Becton Dickinson (BD), USA) at the density of 1.0×104 viable cells/well and incubated for 24 h to allow cell attachment. Following attachment, the medium was substituted with medium supplemented with 5% FBS (150 µl/well) containing the 5-FU, AM, MMT, 5-FU-AM hybrid and 5-FU/AM-MMT composites at equivalent drug concentrations ranging for Hela is 10 to 100 µg/ml and for MCF-7 is 5 to 500 for 72 h. Following treatment, the cells were then washed with PBS and incubated with 150 µl/well fresh medium containing 0.5 mg/ml MTT. The MTT containing medium was removed after incubating for 3 h in dark. The MTT formazan was dissolved in 100 µl/well DMSO and optical density was determined at 570 nm using an ELISA plate reader (Bio-Tek, USA). Cell viability was calculated by the following equation:

$$Cell\ viability(\%)=(A_s/A_{control})\times 100 \quad (3)$$

Where, *As* is the absorbance of the cells incubated with the 5-FU, AM, MMT, 5- FU-AM hybrid and 5-FU/AM-MMT composite hydrogel and *Acontrol* is the absorbance of the cells incubated with the culture medium only. The curve of cell viability (%) against the log concentrations of 5-FU, AM, MMT, 5-FU-AM hybrid and 5-FU/AM-MMT composite hydrogel composites was generated. IC50, the drug concentration at which inhibition of 50% cell growth was observed when compared to control sample, was calculated from the curve of cell viability data.

In Vivo Pharmacokinetics (PK)

Animals and Dosing

10-12 week-old healthy female wistar albino rats weighing 200–250 g were used for the study. The animals were inbred and maintained at the animal house of Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, India under the Registration number 1155/PO/a/07/CPCSEA from the Ministry of Environment and Forest, Government of India, New Delhi and Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India. The animal care, handling and the protocols were approved by CPCSEA recognized local "Institutional Animal Ethics Committee" (IAEC/DPS/SU/1223). The animals were acclimatized at temperature of 25 ± 2°C and relative humidity of 50–60% under 12h light/dark conditions for one week before experiments. All animals were fasted for 24 h before the studies, and water was available ad libitum during the course of study. The animals were arbitrarily distributed into three groups each containing six animals. First group of animals received oral pristine 5-FU, while the second group of animals received 5-FU-AM (dry powder suspension) and third group received 5-FU/AM-MMT composite hydrogels (dry powder suspension). All the formulations were administered orally using a feeding tube attached to a hypodermic syringe at a dose of 5-FU (50 mg/kg) body weight. All animals were observed for their general condition, clinical signs and mortality. The blood samples (approximately 0.3 ml) were collected from the retro orbital plexus under mild diethyl ether anesthesia into the fluoride tube. The time breaks for blood collection were kept at 0 (pre-dose), 1, 3, 6, 9, 12, 24 and 48 h after administration of the drug. Plasma samples were harvested by centrifugation (Heraeus Biofuge Stratos, D-37520, Germany) at 10,000 rpm for 15 min at 5°C. The drug was extracted from plasma samples by solvent evaporation method. 100 µl of plasma were deproteinized with 300 µl of methanol, vortexed for 5 min, centrifuged at 14,000 rpm for 15 min and the step was repeated three times. The supernatants were collected and evaporated in water bath at 60°C, and residues were reconstituted with 100 µl methanol and stored at -20°C for HPLC analysis.

In Vivo Biodistribution Study

The effect of the pristine 5-FU, 5-FU-AM and 5-FU/AM-MMT composite hydrogels on different tissues of rats was evaluated. z Two animals from each group were anesthetized and blood samples were collected from the retro orbital plexus into the plain tube, at the time gap of at 3, 9 and 24 h after drug administration. Immediately after death, carcasses were placed on ice packs and opened by bilateral thoracotomy. The liver, spleen, kidney, heart, lung, thyroid, muscles and blood (in anti clotting vial) were collected. Tissue samples were blotted with paper towel to remove blood, rinsed in saline, blotted to remove excess fluid, weighed, cut into small pieces and then homogenized with 2 ml of phosphate saline buffer. The homogenate was then centrifuged at 14,000 rpm for 30 min at 5°C, the fatty layer was discarded and supernatants were used for drug extraction. The drug was extracted from tissue supernatant by solvent evaporation method. 2 ml of the supernatant were deproteinized with 2 ml of methanol, vortexed for 5 min, centrifuged at 14,000 rpm for 20 min, and supernatants were collected, which was repeated three times. The sample was evaporated at 60°C in water bath and reconstituted with 1.5 ml of the methanol and stored at -20°C for HPLC analysis. Serum from blood was separated and used for biochemical

analysis, total serum protein estimation and SDS-PAGE electrophoresis study.

Drug Quantification by HPLC

The quantification of 5-FU from release media, blood plasma and homogenized tissues was determined by using a validated HPLC method reported in literature with some modifications (Rahman et al.,2008). Briefly, subsequent to the preparation of samples, analysis by high-performance liquid chromatography (HPLC) system consisting of photodiode array detector (Waters Alliance model: 2695 separation module with Waters 2996 Photo diode Array Detector, Waters Corporation, Milford, MA, USA) and a reverse-phase C18 column (Waters X Bridge TM C18 HPLC column with Length = 100 mm, ID = 2.1 mm, Particle size = 5.0 µm, Waters Corporation, Milford, MA, USA) was carried out. 5-FU containing samples were transferred into auto sampler vials, capped and placed in HPLC auto sampler cassettes. Mobile phase employed for the analysis was the mixture of methanol, acetic acid and water (4:0.05:95.95, by volume). The detection wavelength (λ_{max}) for 5-FU was set to be 266 nm and drug concentration in samples was determined using the individual standard curves. For in vitro release, plasma and tissue samples obtained for known concentrations of 5-FU in plasma/tissues processed similarly. The curves were found to be linear with R^2 = 0.9990-9998.

Toxicity Biomarker Assessment and Serum Protein Estimation

The drug toxicity evaluations were carried out by the collection of blood at time intervals of 3, 9 and 24 h throughout the biodistribution experiments. The drug toxicity biomarkers, Alkaline phosphatase (ALP), ALT (Glutamate pyruvate transaminase, SGPT), AST (Serum glutamate oxaloacetate transaminase, SGOT), Total protein (TP) and albumin contents were estimated from rat serum by auto analyzer kit (Span Diagnostics Ltd; Surat, India) using Microlab-300 chemical semi auto analyzer (Merck, Germany) (Detail procedures are given in the supplementary data).

Results and Discussion

Effect of Acrylamide Polymerization on D-spacing of Na^+–MMT's (001) Diffraction Peak

The XRD patterns of MMT, AM, 5-FU-AM, AM-MMT and 5-FU/AM-MMT are plotted in Fig.1. A typical diffraction pattern (001 plan) of Na+-montmorillonite (MMT), with a strong peak corresponding to a basal spacing of 11.28 Å (2θ = 7.83°) was recorded. In the AM-MMT and drug loaded 5-FU/AM-MMT composite hydrogels, this peak was shifted to a lower angle, corresponding to basal spacing of 13.78 Å (2θ = 6.40°) and 14.11 Å (2θ = 6.24°), respectively. The increase in basal spacing implied that the monomer was intercalated into the interlayer of the clay.. This is because the polymerization of acrylamide monomer to polyacrylamide matrix gave a contraction between layers of the MMT. The polymer between the layers of the clay assumedly adopted a flattened conformation. This suggested that in composite hydrogels the clay minerals were intercalated but not exfoliated and superiorly disseminated in polyacrylamide matrix. However, the intensity of the XRD characteristic peaks was decreased in 5-FU/AM-MMT, indicating ordering elasticity of sheet structure caused by the polymeric matrixes formation and the lower restoration of charge density during drug loading.

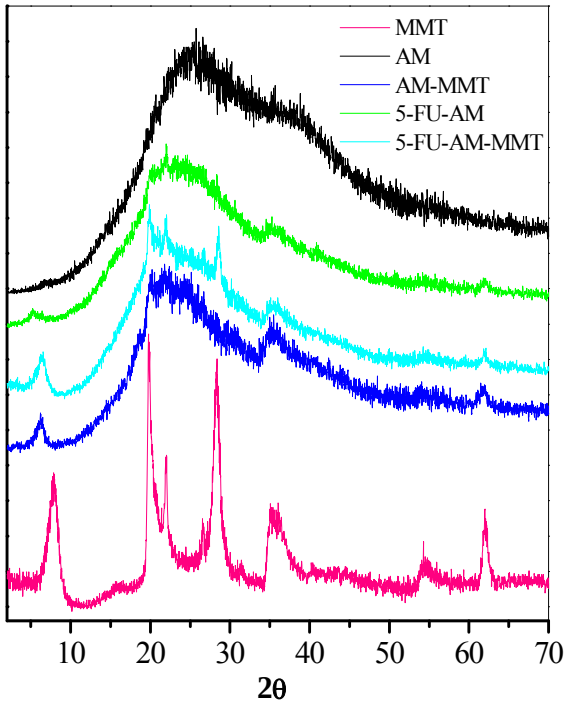

FIG. 1 XRD PATTERNS OF MMT, AM, AM-MMT, 5-FU-AM AND 5-FU/AM-MMT

^{13}C NMR Spectroscopy Analysis

The ^{13}C NMR spectroscopy also indicated the polymerization of acrylamide by the free redical polymerization. In Fig.2 solid-state ^{13}C NMR spectra of composite hydrogel and free AM are depicted. In order to elucidate the spectra of composite hydrogel and define each peak, these spectra were compared with pristine AM. When the spectrum of the 5-

FU/AM-MMT composite hydrogels prepared from AM and MMT is compared with the AM spectra, it becomes clear that the large peak with a shoulder at 44.08 ppm represents the main carbon backbone. The peaks at 183.12 ppm depict the carbon atoms of the amide and carboxylic groups ascribed to carbon having sp3 hybrid orbitals. While composites hydrogel has not shown any peak related to drug due to lesser quantity of drug in composites. Furthermore, the spectrum was similar to that of AM, indicating successfully polymerization of acrylamide by involvement of MMT and formation of composite hydrogels (Sugahara et al., 1990; Dorkoosh et al., 2000; That et al., 2001). (Supplementary data; FT-IR, TGA/DTA and SEM).

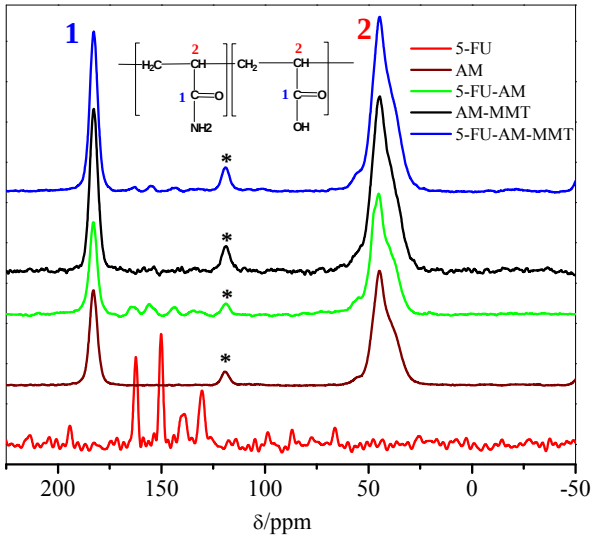

FIG. 2 THE ^{13}C NMR SPECTRA, SPINNING SIDE BANDS ARE MARKED BY ASTERISKS

Swelling Kinetics of Hydrogels

Fig.3 shows swelling kinetics of the AM hydrogels and AM-MMT composite hydrogels in buffers at two different pH. Swelling kinetics of hydrogels in acidic (pH 1.2) and physiological (pH 7.4) media were comparable. The AM hydrogels swell faster (Equilibrium-swollen state=~24 h) than corresponding AM-MMT composite hydrogels. Although elongated time is needed for AM-MMT composite hydrogels to reach their equilibrium-swollen state (> 72 h). The AM-MMT composite hydrogel exhibited much higher degree of equilibrium swelling than that of the corresponding AM hydrogels. The higher degree of swelling observed in case of AM-MMT composite hydrogels was most probably due to the increased hydrophilic character on addition of hydrophilic clay as well as nano layer guided dispersion of cross-linking. The higher swelling observed in the presence of MMT compodite may be due to its properties as functional filler and having higher amount of surface hydrophilic groups on its structural sheets. This in turn increases the osmotic pressure difference between the AM-MMT composite polymeric networks and the external buffer solution and enlargement of the composite hydrogels. The free water penetrates inside the composite hydrogels in order to fill up the inert channels among the composite matrix, contributing to enhanced swelling. Changes in pH of the surrounding liquid had significant effect on swelling process of composite hydrogels. The swelling mechanism of composite hydrogel can be correlated with the diffusional process and the physical–chemical interactions between solvent and matrix.

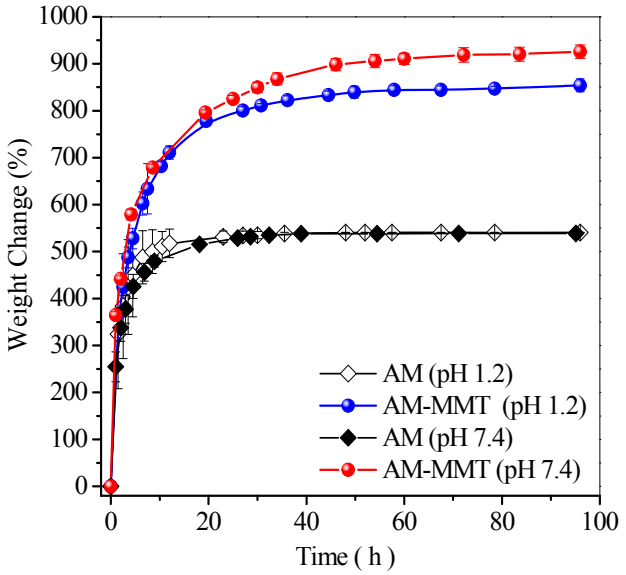

FIG. 3 SWELLING KINETICS OF HYDROGELS IN BUFFERS WITH PH=1.2 AND PH=7.4, DATA REPRESENT MEAN ± SD (N = 3)

TABLE 1 EXPECTED PARAMETERS AND DIFFUSION MODEL FOR SWELLING OF HYDROGELS AT PH 1.2 AND PH 7.4

Exponential Heuristic equation				
Parameter	AM		AM-MMT	
	pH 1.2	pH 7.4	pH 1.2	pH 7.4
r^2	0.9562	0.9691	0.9957	0.9902
n	0.2196	0.2617	0.3271	0.2801
k	0.2815	0.2074	0.1052	0.1201

The water uptake mechanism of the gels was determined through the plotting of the fractional swelling (Wt/W∞) with time, according to Eq. (2), in buffer solutions of pH 1.2 and pH 7.4. The "*n*" values of AM hydrogels and AM-MMT composite hydrogels are listed in Table 1. The plots of ln (W t/W∞) against ln "*t*" are linear (Supplementary data; Fig.S5). From the slopes, value of "*n*" could be obtained. The values of "n" in pH 1.2 and pH 7.4 solutions for AM hydrogel and AM-MMT are 0.21-0.32, which indicates that the transport of water in the hydrogel is due to water diffusion partially through a swollen polymeric matrix

and water filled in clay layers of the composite hydrogels. The swelling mechanism may be Fickian diffusion, which means that the diffusion of solvent controls the swelling process.

In Vitro Drug Release Profiles

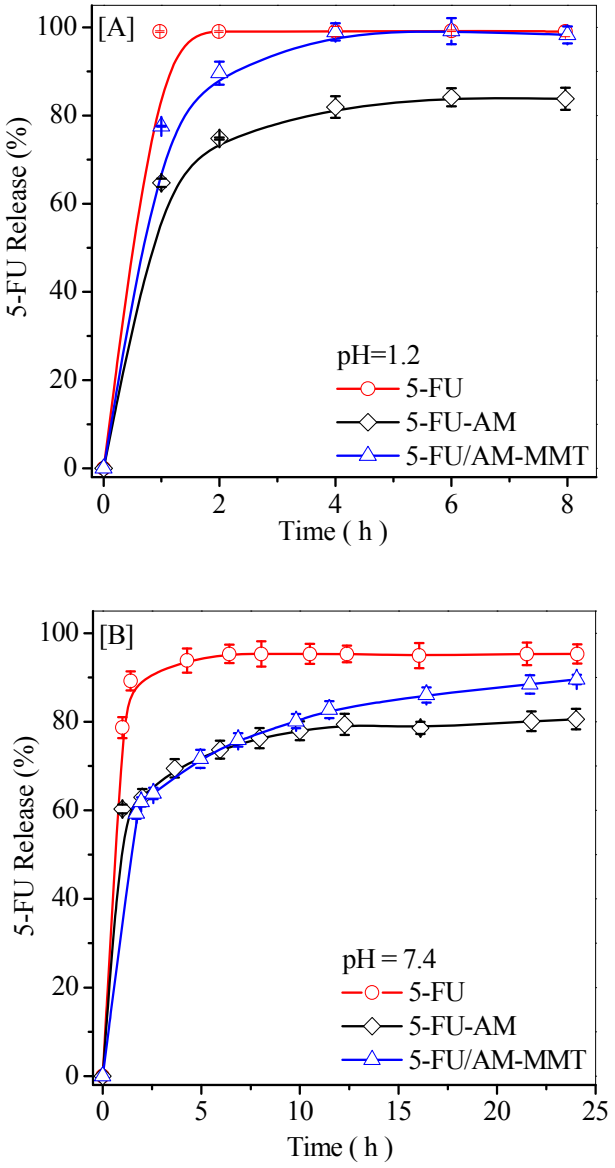

FIG. 4 *IN VITRO* DRUG RELEASE PROFILES OF 5-FU AT 37±0.5 °C (A) PH 1.2 AND (B) PH 7.4 DATA REPRESENT MEAN ± SD (N = 3)

The drug release patterns of 5-FU-AM and 5-FU/AM-MMT composite hydrogels in buffer solution pH of 1.2 and 7.4 are given in Fig. 4A and B. The release of drug after 8 h from the AM and AM-MMT composite hydrogel matrices at pH 1.2 is ~83% and ~98 %, respectively. While the drug release after 24 h from the AM and AM-MMT composite hydrogel matrices at pH 7.4 is ~ 80% and ~90%, respectively. The negative charge on clay decreases with the decrease in pH, which indicates that drug, binds loosely to clay and strongly to polyacrylamide and therefore it is released slowly from AM after 8 h at pH 1.2. The presence of MMT in hydrogel influenced the interaction of drug with polyacrylamide and accelerated the release of drug from 5-FU/AM-MMT composite hydrogels. Thus, drug release was comparatively faster from the composite hydrogels than AM hydrogels at both pH. The presence of MMT in the composite hydrogels is also reported to result in protection from dietary toxins, microbial toxins and promote mucoadhesiveness of the carrier polymers by interacting with the gastric and intestinal mucosa (Dong and Feng, 2005; Feng et al., 2009). The release of drug from the composite hydrogels could be tuned by controlling the amount of MMT in the composite hydrogels.

To understand the release mechanism of drug molecules from the 5-FU-AM and 5-FU/AM-MMT composite hydrogel carriers, the parabolic diffusion equations and Elovich kinetic models were employed (Table 2) and (Supplementary data; Fig.S7). The best linearity was obtained in both the model (R^2=0.9623–0.9968). Thus, the kinetics of 5-FU release was governed by diffusion controlled exchange and partial diffusion through a swollen matrix of the hydrogels.

TABLE 2 LINEAR CORRELATION COEFFICIENT (R^2) OF THE DIFFUSION KINETIC MODELS APPLIED TO 5-FU RELEASE FROM PRISTINE HYDROGELS AND COMPOSITE HYDROGELS (RELEASE DATA WAS CONSIDERED FROM INITIAL 4 H AT PH 1.2 AND INITIAL 8 H AT PH 7.4)

Samples	Elovich equations		Parabolic diffusion equations	
	pH 1.2	pH 7.4	pH 1.2	pH 7.4
5-FU-AM	0.9907	0.9811	0.9623	0.9818
5-FU/AM-MMT	0.9947	0.9968	0.9708	0.9900

In Vitro Cell Viability of Composite Hydrogel

Fig.5 shows a comparison of *in vitro* viability of HeLa (Fig.5a) and MCF-7 cells (Fig. 5 b) after exposure to 5-FU, AM, MMT, 5-FU-AM, and 5-FU/AM–MMT composite hydrogel at the different concentrations after 72 h of incubation. The 5-FU, 5-FU-AM and 5-FU/AM–MMT showed dose-dependent reduction in cell viability in HeLa and MCF-7 cells. The in vitro effect of 5-FU, 5-FU-AM and 5-FU/AM–MMT was quantitatively evaluated by IC50 defined as the drug concentration at which 50% cells were killed at a given time period. From value of IC50, it has been concluded that the HeLa cells are generally less susceptibility to 5-FU, 5-FU-AM and 5-FU/AM–MMT as compared to MCF-7 cells. Therefore, MCF-7 cells were considered to be extremely sensitive to MMT and AM. However, it was advocated based on results that 5-FU could preserve its antitumor effectiveness subsequent to its absorption in composite hydrogel.

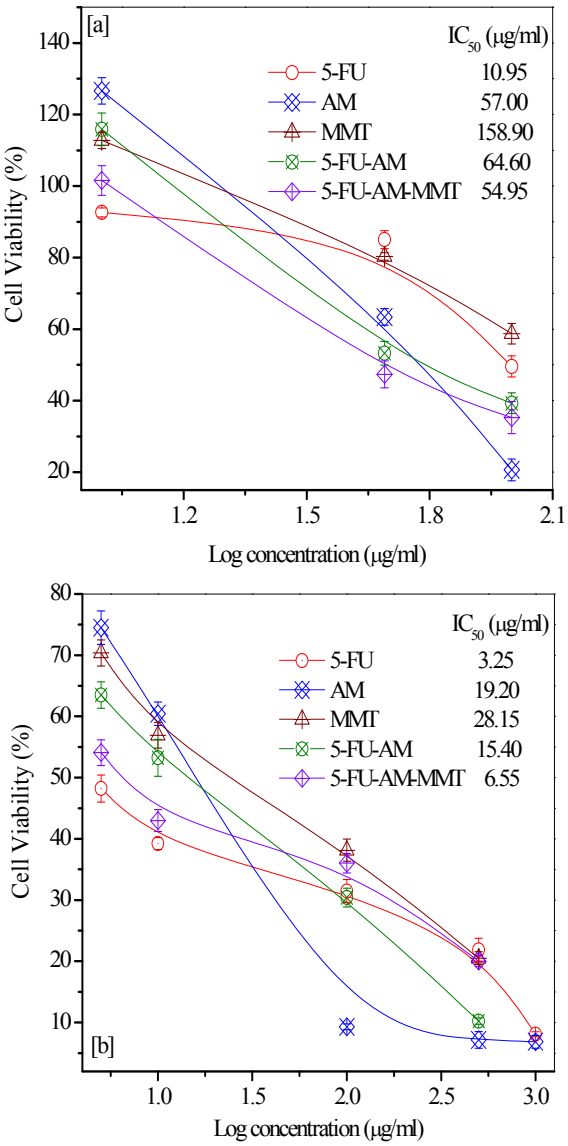

FIG. 5 *IN VITRO* CELL VIABILITY OF (A) HELA AND (B) MCF-7 CANCER CELLS AFTER 72 h OF INCUBATION WITH 5-FU, MMT, AM,AM-MMT AND 5-FU/AM-MMT. THE CONCENTRATION RAGE FOR HELA AND MCF-7 WAS 10-100 μG/ML AND 5-500 μG/ML OF 5-FU DOSES, RESPECTIVELY; RESULTS ARE SHOWN AS MEANS ± SD (N = 6).

In Vivo Pharmacokinetics (PK)

The plasma concentration level of 5-FU was determined after a single oral administration of pristine drug and drug in AM/AM-MMT composite hydrogels at a dose of 50 μg/kg 5-FU equivalent in female wistar rats (Fig. 6) over a period up to 48 h. The key PK parameters have been analyzed and the results are listed in Table 3, including Cmax (in μg/ml) and Tmax (h)–the maximum drug concentration encountered after the drug administration and the time at which C max is reached, MRT (h)–the mean residence time of the drug in the plasma and AUC 0–∞ (μg h/ml)–the total area under the curve that represents the *in vivo* therapeutic effects of drug. Some advantage of AM and AM-MMT nanocompostie hydrogels could be concluded from PK data. The MRT and Tmax of the drug in the plasma were increased to some extent in formulated drug. Therefore, therapeutic effective period for the 5-FU/AM-MMT nanocompostie hydrogels was little longer than that for the free 5-FU, while the $AUC_{0-\infty}$ and Cmax value of formulated and pristine drug were not significantly different. It was observed that the 5-FU was liberated from composite hydrogel in a controlled mode with increment in blood circulation time as well as drug concentration peak in the rats.

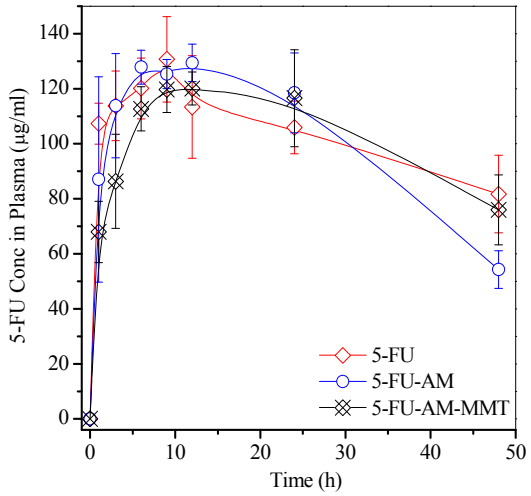

FIG. 6 TIME PROFILES OF RELATIVE PLASMA CONCENTRATIONS OF 5-FU AFTER ORAL ADMINISTRATION TO FEMALE WISTAR RATS WHEN FORMULATED IN THE AM AND AM-MMT COMPOSITE HYDROGELS AS COMPARED TO PRISTINE 5-FU, RESULTS ARE SHOWN AS MEANS ± SD OF SIX ANIMALS PER GROUP

TABLE 3 PHARMACOKINETICS (PK) OF PRISTINE 5-FU, 5-FU-AM HYDROGELS AND 5-FU/AM-MMT COMPOSITE HYDROGELS IN FEMALE WISTAR RATS AFTER SINGLE ORAL ADMINISTRATION OF SAME DRUG DOSE; DATA REPRESENT MEAN OF SIX ANIMALS PER GROUP

PK parameters	5-FU	5-FU-AM	5-FU/AM-MMT
C_{max} (μg/ml)	130.70±15.6	129.40±16.7	120.09±13.8
T_{max} (h)	9	12	12
$AUC_{0-\infty}$ (μg h/ml)	2801.44± 336	2934.80±381	2724.80±313
MRT (h)	13.50	13.33	14.25

Biodistribution Study

The distribution of 5-FU in solid tissues such as liver, spleen, kidney, heart, lung, thyroid and muscles was investigated at three time points: 3, 9 and 24 h. The results are shown in Fig. 7 for (a) pristine 5-FU, (b) 5-FU-AM and (c) 5-FU/AM-MMT composite hydrogel. It was seen that MMT incorporation in AM matrix leads to significant changes in the biodistribution of the drug. The peak concentrations of drug were detected after 3 or 9 h, for pristine 5-FU administration. Whilepeak concentrations of drug were detected after at 3 and 9 or 24 h for the 5-FU-AM and 5-FU/AM-MMT composite hydrogels, respectively. MMT

incorporation in AM hydrogels leads to delay in the time of the peak concentrations and their magnitude reduces, which may imply reduced side effects and thus having clinical significance. Such an advantage of MMT incorporation may be attributed to the slower penetration of the drug into the tissues. For 5-FU, the highest concentration was found in Thyroid (781.2± 203.6 μg/g at 3 h) followed by liver (650.3± 34.7 μg/g at 3 h), spleen (640.2±12.5 μg/g at 3 h), kidney (573.1±51.0 μg/g at 3 h), heart (537.6±26.2 μg/g at 9 h), lung (480.7± 42.5 μg/g at 3 h), and muscles (445.9±115.8μg/g at 3 h). On the contrary, the peak concentration in these tissues for the 5-FU/AM-MMT composite hydrogels dropped significantly. The 5-FU-AM hydrogel presented a little lower 5-FU peak levels than the free drug in these tissues. There was significant variation in concentration and time of distribution of drug between pristine drug and drug loaded composite hydrogels in these tissues up to 24 h after administration of a single oral dose. It was clearly observed that the drug was released from composite hydrogel in a controlled manner with effective distribution in the various active tissues of the rat.

Toxicity Biomarkers Assessment and Serum Protein Estimation

The results of hepatotoxicty in terms of increase in enzyme levels of alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT) and alkaline phosphatase (ALP) are as shown in Fig 8. Elevated levels of SGPT/SGOT and ALP activity can display the degree of hepatotoxicity. The serum samples from pristine 5-FU, and 5-FU/AM-MMT composite hydrogel treated rats, when analyzed for SGPT/SGOT and ALP at different time intervals as markers of liver toxicity which showed significant increase in the activity in 5-FU treated rats group as compared to 5-FU/AM-MMT composite hydrogel treated rat groups. During the first three hours, activities of SGPT/SGOT and ALP were significantly higher in all the treated rats than those in the control group which consequently declined with time in 5-FU/AM-MMT composite hydrogel groups compared to pristine 5-FU treated group. The elevated levels of serum marker enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane of liver cells. Serum ALP activities on the other hand are related to functioning of hepatocytes, whose increase in serum is due to increased synthesis in the presence of higher biliary pressure. Any decrease in the activity of the above enzymes would indicate reversal of toxicity of liver. The results of serum protein analysis from 5-FU, and 5-FU/AM-MMT composite hydrogel treated rats are shown in Fig.9. In the initial hours serum protein levels are slightly higher in the all treated rats than those in the control group. The elevated serum ALP activities with an increase in the total plasma protein content have suggested that membrane and cell architecture of liver are damaged by 5-FU, which has been confirmed by histopathological examination. Qualitative analysis of the total proteins by SDS-PAGE results did not indicate any structural changes or shearing of proteins in total serum after treatment with 5-FU and 5-FU/AM-MMT composite hydrgels. Therefore, 5-FU and its formulation did not affect the total serum protein configurations (Supplementary data; Fig.S10).

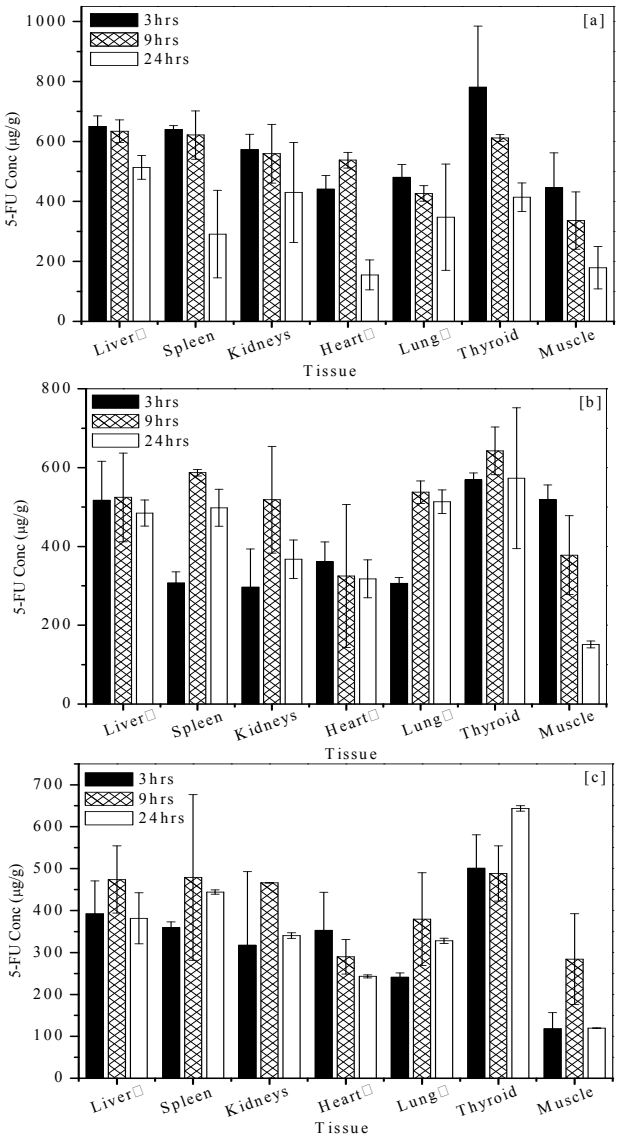

FIG. 7 THE 5-FU LEVELS (μg/g) IN LIVER, SPLEEN, KIDNEY, HEART, LUNG, THYROID AND MUSCLES AFTER SINGLE ORAL DOSE ADMINISTRATION AT 50 MG/KG EQUIVALENT DRUG OF (a) FREE 5-FU, (b) 5-FU-AM HYDROGEL AND (c) 5-FU/AM-MMT COMPOSITE HYDROGELS, RESULTS ARE SHOWN AS MEANS±SD

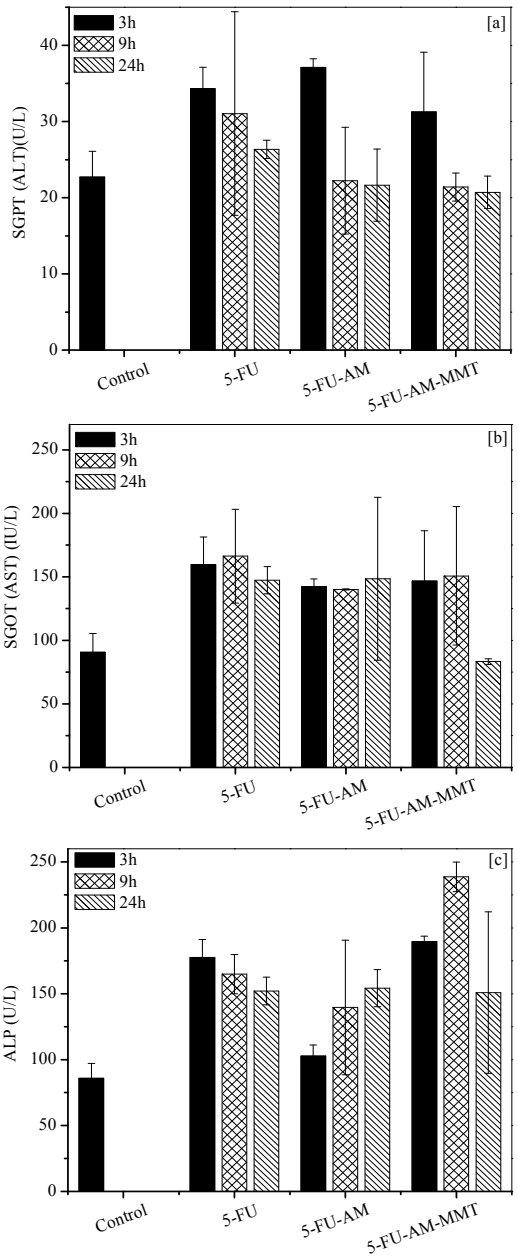

FIG. 8 (a) SGPT (ALT), (b) SGOT (AST), AND (c) ALP LEVELS IN PLASMA AT DIFFERENT TIME GAPS AFTER ORAL ADMINISTRATION OF 5-FU TO WISTAR RATS, DATA REPRESENT MEAN ± SD

Conclusion

Polyacrylamide and Na^+-montmorillonite composite hydrogel were prepared by free radical polymerization, characterized and used as anticancer drug carrier. Swelling kinetics experiments showed that the degree of swelling was higher in composite hydrogel as compared to pristine hydrogel and introduction of the hydrophilic MMT improved the water absorbance of composite hydrogel. *In vitro* release study of drug showed that the release from composite hydrogel was controlled with time and partial diffusion through swollen matrix of the composite hydrogel. *In vitro* cytotoxicity experimental results revealed that after incorporation of 5-FU in composites hydrogel it was safe to used and its antitumor effectiveness remain unchanged. *In vivo* pharmacokinetics, biodistribution, toxicity biomarkers and serum protein analysis results indicated that AM-MMT composite hydrogel were better carrier 5-FU as compared to pristine drug. Our results indicated that clay based composite hydrogel has excellent prospective as drug carriers for the treatment of cancer.

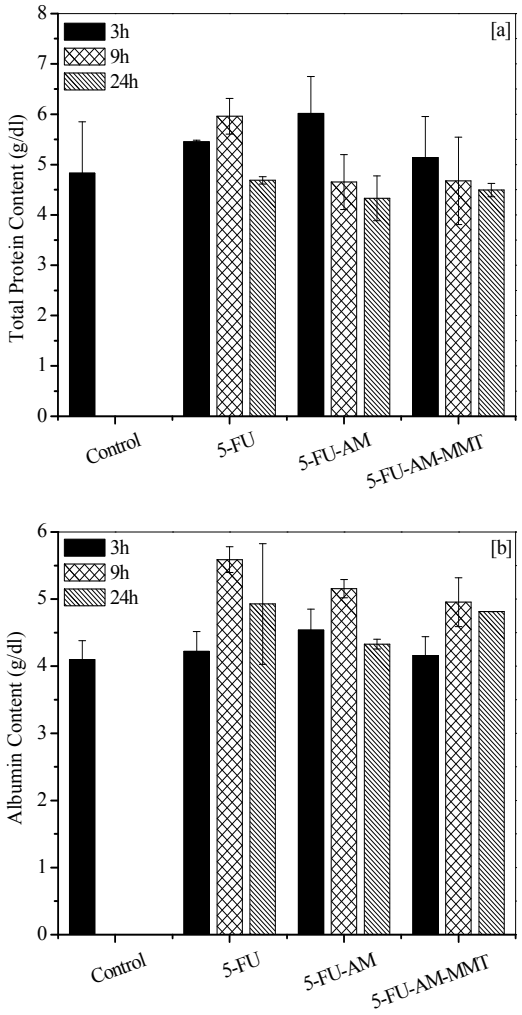

FIG. 9 ANALYSIS OF (A) TOTAL PROTEIN CONTENT, AND (B) ALBUMIN CONTENT IN SERUM AT DIFFERENT TIME GAPS AFTER ORAL ADMINISTRATION OF 5-FU TO WISTAR RATS, RESULTS ARE SHOWN AS MEANS ± SD

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version, at…

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Supplementary Data

S1.Synthesis of AM hydrogel and AM-MMT composite hydrogel

Cross-linked polyacrylamide hydrogel are formed by

the polymerization of acrylamide monomer in the presence of smaller amounts of bis-acrylamide. Bis-acrylamide essentially has two acrylamide molecules linked by a methylene group, and is used as cross-linking agent. Acrylamide monomer is polymerized in a head-to-tail fashion into long chains and occasionally bis-acrylamide molecules are built into the long chain, thus introducing a second site for chain extension. The polymerization of acrylamide monomer is free radicals catalysis, and is initiated by the addition of APS and TEMED. The TEMED catalyses decomposition of persulphate ion gives free radical (Wilson and Walker). Composites hydrogel were shaped by conglomeration and some interaction with AM in MMT interlayer gallery. AM-MMT composite hydrogel were synthesized by free radical polymerization in distilled water containing of Na^+-MMT in optimized ratio of AM to MMT (6:1%, w/w), APS and TEMED were added as the initiator and accelerator, respectively. The hydrogel without MMT were translucent and MMT containing hydrogel decreased in transparency in the AM-MMT composites and the dispersion of MMT exhibited uniformity.

S2. FT-IR spectra of MMT, AM, AM-MMT, 5-FU, 5-FU-AM and 5-FU/AM-MMT

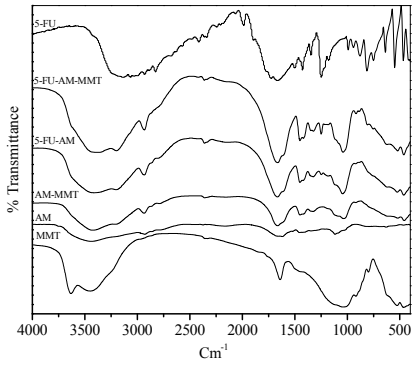

Fig. S1.

The characteristic FTIR spectra of the MMT, AM, 5-FU, AM–MMT, 5-FU-AM and 5-FU/AM-MMT composite hydrogel are presented in Fig.S1. The spectrum of MMT revealed the characteristic absorption bands (Yi and Zhang, 2007; Joshi et al., 2009). The absorption peak at 1666 cm^{-1} was attributed to –CO-NH_2 on acrylamide and the characteristic peak at 1431 cm^{-1} was attributed to –CH_2 – (Wu, et al., 2003). The asymmetric vibration of C–H band at 2930 cm^{-1} of AM in spectrum AM-MMT and 5-FU/AM-MMT indicated that the composite hydrogel were polymerized and cross-linked by the breaking the C = C bonds (Kevadiya et al., 2011). The -NH_2 stretching band of AM consisted of a broad band centered at 3437 cm^{-1} and 3200 cm^{-1}, which shifted to low-frequency in 5-FU-AM, AM-MMT and 5-FU/AM-MMT. In AM-MMT and 5-FU/AM-MMT composite hydrogel, absorption peaks of –CO-NH_2 and –CH_2 – shifted to low-frequency compared to pristine AM because the -OH group on the surface of MMT participated in the reaction. The two additional bands of 5-FU at 1418 and 807 cm^{-1} was confirmed successful 5-FU absorption in matrix of AM and AM-MMT composite hydrogel.

S3. (A) TGA and (B) DTA pattern

Fig.S2.illustrate the TGA pattern the DTA pattern of dried MMT, AM, AM-MMT and 5-FU loaded composite hydrogel. The TGA curve of pristine MMT showed weight loss in four steps at the temperature around ~100°C, 270°C, 430°C, and 550-700°C. The first weight loss and endothermic peak at ~100 °C in MMT corresponded to the loss of adsorbed water. The weight loss at 430°C was due to dehydroxylation of MMT (Kevadiya, et al., 2011).

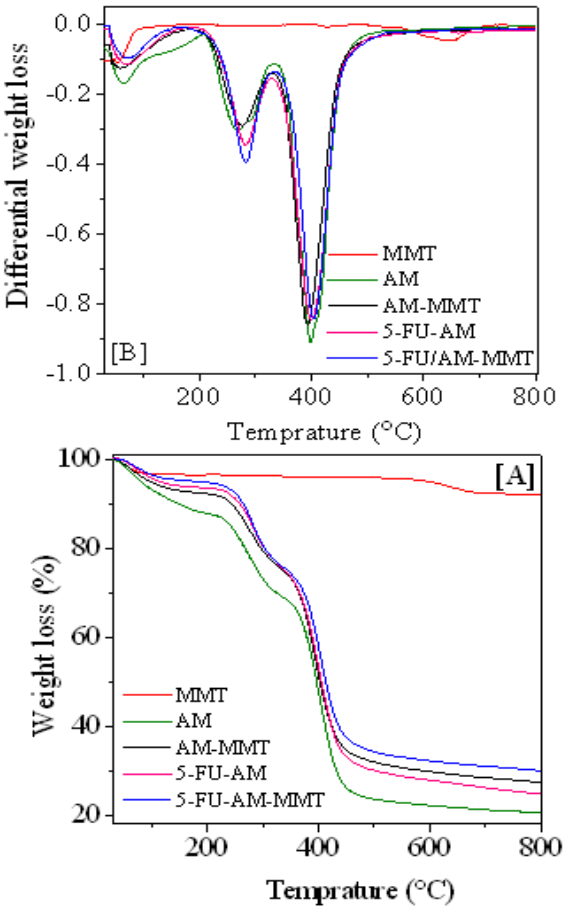

Fig. S2.

The TGA curves of AM, 5-FU-AM, AM-MMT and 5-FU/AM-MMT composite hydrogel exhibited weight loss at the temperature around 65-75°C, 270-290°C, and 400- 410 °C. The first weight loss at around 65-

75°C was due to the free water evaporation from AM. The second weight loss at the temperature around 270-290°C were due to the removal of 5-FU from different cross liking patterns of the AM-MMT and disintegration of AM structure. The third strong endothermic peaks were due to weight loss at the temperature around 400-410°C, corresponding to the complete degradation of AM from 5-FU-AM and 5-FU/AM-MMT composite hydrogel.

S4. SEM pictures of freeze-dried (A) pristine MMT (B) AM (C) AM-MMT (D) 5-FU/AM-MMT

The SEM images of the pristine MMT, AM, AM-MMT and 5-FU/AM-MMT are shown. MMT (Fig.S3A) exhibits layered structure with platelet morphology consisting of stacked silicate sheets which are ~ 1 nm thick and 200 nm long. The cross-sectional view of the freeze-dried AM hydrogel reveals porous nature of the matrix (Fig. S3B). Fig. S 3C reveals vastly porous matrix of AM-MMT composite hydrogel with uniformly interconnected channel-like structures. Fig.S3D shows the surface morphology of 5-FU/AM-MMT composite hydrogel after drug loading and slight compacting of the matrix structure. Pristine MMT based formulations used in oral administration are limited because of their burst drug release and limited capacity of swelling in the body fluids. Because of these limitations, MMT with polyacrylamide matrixes have been synthesized.

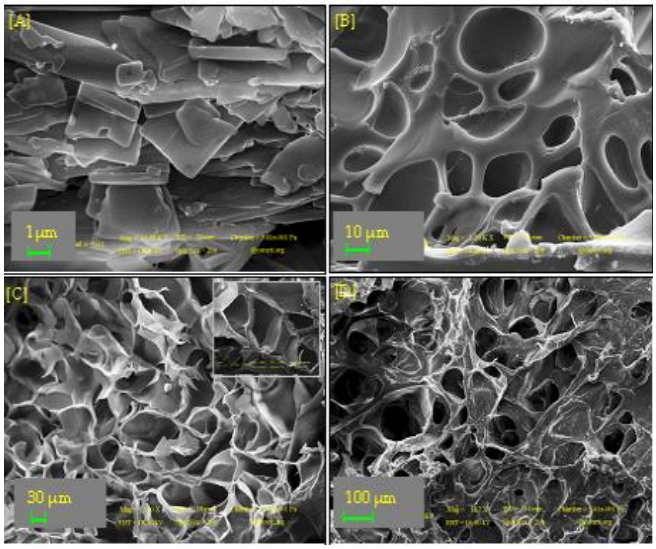

Fig S3

S5 Effect of drug concentration on 5-FU loading in hydrogel Data represent mean ± SD (n = 3)

To study the effect of drug concentration on the 5-FU absorption into AM hydrogels and AM-MMT composite hydrogels, reactions were carried out at different initial concentrations of 5-FU (up to solubility limit of drug). 100 mg of AM hydrogels and AM-MMT composite hydrogels were added in 10 ml aqueous solution of 5-FU containing different amount of 5-FU and kept for five days at room temperature. The free drug solutions were filtered and quantified by UV–visible spectrophotometer.

Absorption of 5-FU into AM and AM-MMT composite hydrogel was highly concentration dependent (Fig S4). As the initial concentration of 5-FU increased, the absorption quantity of 5-FU also increased due to the concentration gradient and swelling of hydrogel. However, at highest solubility of 5-FU (~9 mg/ml), the absorption of 5-FU was ~118 mg of 5-FU/g in AM and ~146 mg of 5-FU/g in AM-MMT composite hydrogel. It means the absorptions of 5-FU was straight propositional to degree of swelling kinetics of hydrogel.

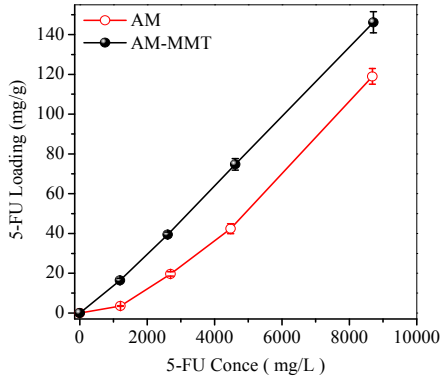

Fig. S4.

S6. ln (W_t/W_{inf}) versus time ln't' graph for AM hydrogel and AM-MMT Nanocomposite hydrogel at pH 1.2 and pH 7.4

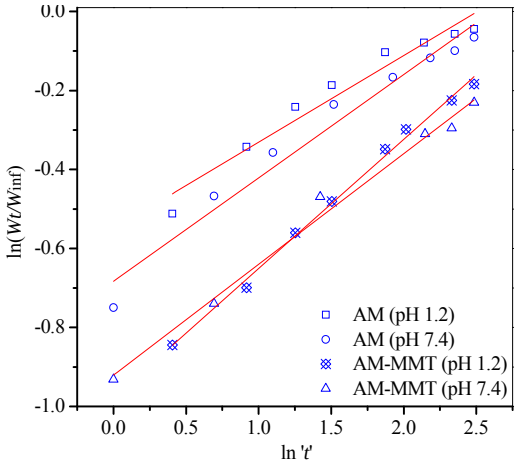

Fig. S5.

S7. HPLC Standard plot of in vitro drug release (Concentration range of 5-FU is from 0.09 ppm to 500 ppm)

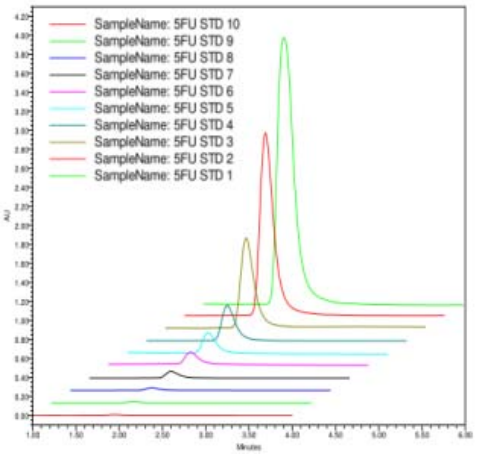

Fig. S6

S8. Fitting the 5-FU release data to (A) Elovich and (B) parabolic diffusion equations

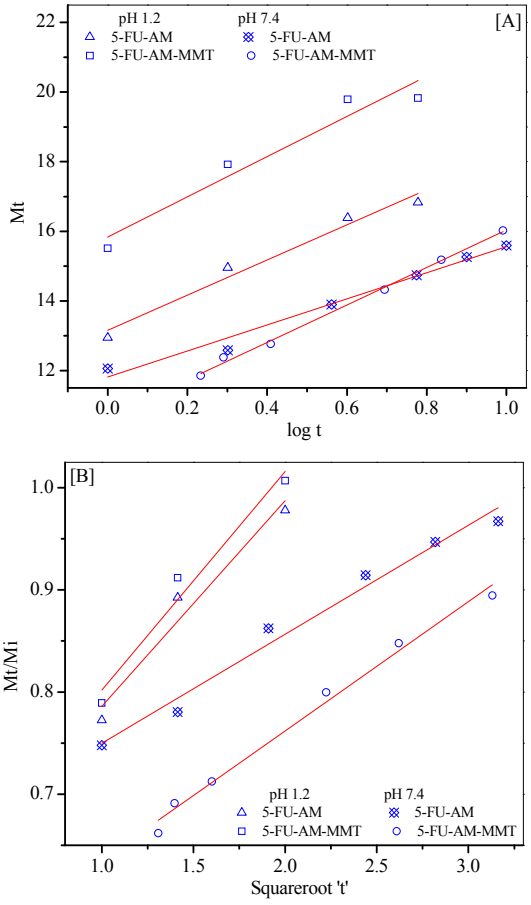

Fig. S7

S9. In vitro evaluation of gastrointestinal microflora (Lactobacillus spp) sensitivity

S9.1. Well-diffusion assay

The sensitivity of gastrointestinal microflora (*Lactobacillus spp*) to the drug loaded or pristine acrylamide and composite hydrogel was assayed by well-diffusion antagonism method. 1 % agar-agar was poured in a petri dish and allowed to solidify. After complete solidification, Nutrient broth (Himedia, Mumbai) supplemented with 0.75% agar-agar and MRS broth supplemented with 0.75% agar-agar medium containing approx 1×10^7 to 1×10^8 cells per ml (McFarland Standard 0.5) with optical density of 0.132 at 625 nm was layered over it. 7-mm wells were punched and 75 µL of the drug loaded suspensions and pristine composite hydrogel were placed in concentration range of 200 µg/ml, 500 µg/ml and 1000 µg/ml of either drug or equivalents to pristine carrier of hydrogel in each well. The plates were incubated at 37°C for 24 h. The sensitivity of gastrointestinal microflora to drug was evaluated by measuring the transparent halo circles around the wells after incubation. That is to say, when an agent has sensitivity to gastrointestinal microflora, the halo circle is formed along the periphery of the composite hydrogel. All glass wares used in this study were sterilized in an autoclave at 121°C, 15 psi for 30 min before each experiment to exclude any possible microbial contamination.

S9.2. Preparation of bacterial cells for SEM

Preparation of samples for SEM involves three separate processes; fixation, dehydration and coating with heavy metals. Bacterial suspensions at appropriate dilutions were prepared from cultures growing in petri dishes containing bacteria treated with composite hydrogel by well-diffusion method. 15 µl of the bacterial suspension was applied onto the surface of metal stub and air dry for 30 min at room temperature. The sample was washed thrice with phosphate buffer solution (pH 7.0) and fixed with 2.5 % glutaraldehyde for 30 min. The sample was then washed four times with double distilled water and incubated with 0.1% OsO_4 for 30 min. Finally, it was washed with 0.25 %, 0.50 %, 0.75 % and 100 % of ethanol in Milli-Q water and air dried before visualizing under scanning electron microscope (Bayer, et al., 1974).

S9.3. In vitro gastrointestinal microflora sensitivity assay

The gastrointestinal microflora (*Lactobacillus spp*) have a number of significant functions including epithelial protection and metabolism of bilirubin, intestinal mucins, pancreatic enzymes, fatty acids, bile acids, cholesterol and steroid hormones. Further more gastrointestinal bacteria function to process nutrients, regulate intestinal angiogenesis and work with the immune system (Stringer, et al., 2009). The well-diffusion test results of 5-FU/AM and 5-FU/AM-MMT composite hydrogel are shown in Fig.S8. All the

samples failed to produce halo circles around wells bearing the tested *Lactobacillus spp*, reflecting that the gastrointestinal microflora was not inhibited by these materials. The results was indicated that the composite hydrogel are safe carriers for 5-FU. Further confirmation of carrier inertness was carried out by SEM analysis (Fig.S9) which showed no any incidence of *Lactobacilli* cell wall damage as all the cells appeared normal.

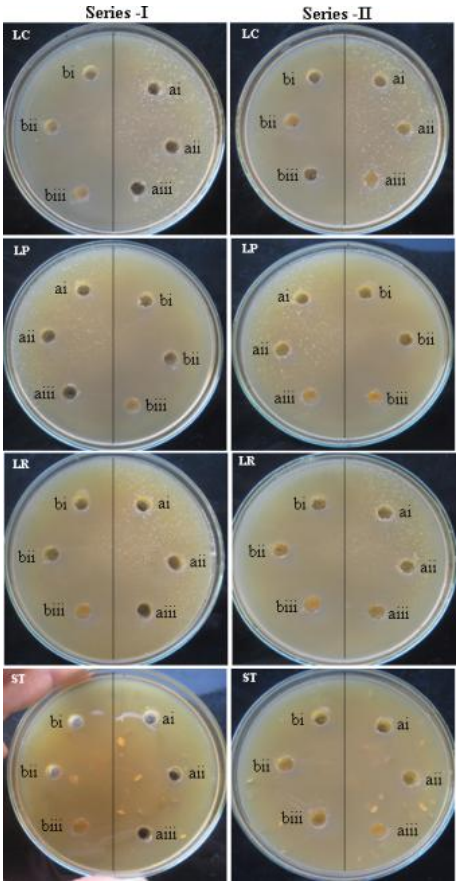

Fig. S8 *Gastrointestinal microflora sensitivity test Series –I: 5-FU-AM (a) and AM (b), Series –II: 5-FU/AM-MMT (a) and AM–MMT (b), the concentrations of hydrogel is indicated by i=200 μg/ml, ii=500 μg/ml and iii =1000 μg/ml with respective groups. Where, LC:Lactobacillus casei, LP:Lactobacillus plantarum and LR:Lactobacillus rhamnosus*

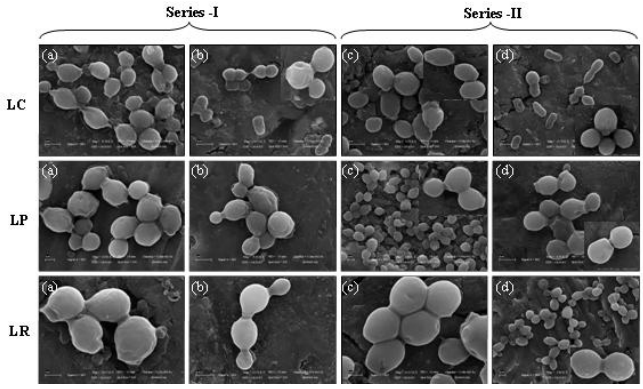

Fig. S9 *SEM images of gastrointestinal microflora after treatment with pristine and drug loaded hydrogel, Series–I treated with AM (a) and 5-FU-AM (b) and in Series –II treated with AM-MMT (c) and 5-FU/AM-MMT (d), Where, LC: Lactobacillus casei, LP: Lactobacillus plantarum and LR: Lactobacillus rhamnosus*

S10.Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT)

AST (SGOT) powder reagent was reconstituted with the 3 ml of deionized water. 1000 μL of reagent was mixed with 100 μL of serum. After mixing well mixture was incubated for 60 sec (37° C) and absorbance was measured at 340 nm. Two additional readings were measured at 1 min interval. The mean absorbance change per min was calculated. ΔA/min was multiplied by factor 1768 (Henry et. al. 1974).

$$Activity\ of\ SGOT\ (IU/L)=\Delta A/min\times 1768$$

S11. Estimation of Serum Glutamate Pyruvate Transaminase (SGPT)

In an eppendroff tube, 800 μl of reagent 1 (125 mmol/L Tris buffer pH-7, 680 mmol/L L-alanine, ≥ 200 U/L LDH) and 200 μl of reagent 2 (97 mmol/L α-ketogutarate, 1.1 mmol/L NADH) were mixed. After 25 sec 100 μl of serum sample was added. After incubation of 60 sec (37° C) the changes in absorbance per min (ΔA/min) for 3 min was measured at 340 nm. And result was calculated as follows (Henderson and Moss 2001).

$$Activity\ of\ SGPT\ (U/L)=\Delta A/min\times 1746$$

S12. Estimation of Alkaline Phosphatase (ALP)

In an eppendroff, 400 μL Reagent 1(1.4 mol/L Diethanolamine pH 10.2, 0.625 mmol/L Magnesium chloride) and 100 μl Reagent 2 (50 mmol/L p-Nitrophenylphosphate) were mixed and allowed for 25 sec at room temperature. Then 10 μL of serum sample was added and incubated for 50 sec. Absorbance was measured at 405 nm per min (ΔA/min) for 180 sec. Result was calculated by using following formula (Scherwin et al., 2003).

$$Activity\ of\ ALP\ (U/L)=\Delta A/min\times 2750$$

S13. Estimation of Albumin

The eppendroff tube contains 10 μL of serum sample; standard tube contains 10 μL of standard Reagent 2 (4 g/dl albumin standard). In blank, standard and sample tubes 1000 μL of Reagent 1 (37 mM/L Succinic acid, 0.15 mM/L Bromocresol green, 1 mM/L Sodium hydroxide, and Buffer pH 3.68) was added. Tubes were mixed well and absorbance was measured after 1 min incubation (15-30°C) at 630 nm. Results were calculated by using following formula (Kaplan et al., 1983).

$$Albumin\ (g/dL)=Absorbance\ of\ Test/Absorbance\ of\ standard\ Conversion\ Factor$$

Albumin Concentration in g/L=albumin concentration in g/dL×10

S14. Estimation of Total Protein (TP)

The eppendroff tube contains 10 µL of serum sample; standard tube contains 10 µL of standard Reagent 2 (6.5 g/dl Protein standard). In blank, standard and sample tubes 1000 µL of Reagent 1 (7 mM/L Copper sulphate, 200 mM/L Sodium hydroxide, 20 mM/L Sodium potassium tartrate) was added. Tubes were mixed well and absorbance was measured after 5 min incubation (37° C) at 578 nm. Results were calculated by using following formula (Koller et al., 1984).

Total protein (TP) concentration (g/dL) = Absorbance of Test / Absorbance of standard
Conversion Factor

Total Protein concentration in g/L = Total Protein concentration in g/dL×10

S15. SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

S15.1. Method

The gel plates were prepared in a casting stand and the bottom of the glass plates were sealed using acrylamide mixture (2ml D/W + 4ml 30% acrylamide + 500 µl APS + 20 µl TEMED). Acrylamide solution was poured gently from the sides, the assembly was holded erect so that the acylamide solution forms the straight layer at the bottom. Acrylamide was allowed to solidify. The process was repeated similarly to seal the two sides; one after the other.

S15.2.Casting of the resolving gel

The following components of 10 % resolving gel solution were mixed in a clean dry beaker. The solution was immediately transferred between the glass plates using a pipette. Water saturated isobutanol was layered onto the solution. The gel was allowed to polymerize for 30-40 min.

Component	*Volume*
30 % Acrylamide	6.7 ml
Tris-HCl (8.8 pH)	5 ml
D/W	7.9 ml
10 % SDS	0.2 ml
10 % APS	0.2 ml
TEMED	0.008 ml

S15.3. Casting of the stacking gel

After polymerization, casting stand was inverted over sink & isobutanol was rinsed out. Residual isobutanol was washed using D/W & drained completely. The stacking gel solution was prepared by mixing the components in a clean dry beaker.

Component	*Volume*
30 % Acrylamide	1.7 ml
Tris-HCl (pH-6.8)	1.25 ml
D/W	6.8 ml
10 % SDS	0.1 ml
10 % APS	0.1 ml
TEMED	0.01 ml

The solution was immediately transferred between the glass plates using a pipette. The comb was instantly inserted from the top, slowly enough to avoid bubble formation below the teeth. The gel was allowed to polymerize for 30-40 min.

S15.4. Sample preparation, loading of the sample and the gel run

After polymerization of stacking gel, the comb was pulled out straight upwards. The upper tank was filled with 1X tank buffer & it was ensured that wells were saturated with this buffer before loading the samples. The lower reservoir was filled with running buffer.

S15.5. Sample preparation

Samples were prepared for SDS-PAGE by mixing 40µl (60 µg) of each of the sample with 10 µl loading buffer and then vortexing it to mix it well. Samples were loaded using micro-pipette. Tracking dye could also be loaded in the wells prior to loading the samples. The apparatus was connected with lid. The voltage of 150V was applied till the samples were run through the stacking gel. Once tracking dye entered the resolving gel, the voltage was increased to 200V. The samples were allowed to run through the resolving gel till the tracking dye approached the lower unit of the resolving gel. The current was switched off.

S15.6. Post runs disassembly

Lid and electric posts were detached and the tank buffer was removed by pouring it out from the reservoir. The glass plate-spacer assembly was unscrewed from the rest of the apparatus. The two glass plates were separated so that the gel was on the top place of the glass plates. The stacking gel was sliced off on a clean level surface using a single edged razor blade & discarded using a paper towel; slightly rinsed with a stream of D/W. The plate was tilted upward so that the gel was not slide off by chance. The glass plates were inverted over the staining tray.

S15.7. Staining and Destaining

The gel was incubated in staining solution (0.05 % Coomassie Brilliant Blue) for overnight & then

destained by changing the destain solution (50 % Methanol + 10% Glacial Acetic Acid) 3-5 times (incubated every time for approximately 40-60 min.). The destaining process was continued until the stained protein bands were clearly visualized over a clear gel background.

S16. The qualitative analysis of total serum protein (TP) by SDS-PAGE method of female wistar rats treated with pristine 5-FU (line no. 1, 2 for 3 h, line no. 7 , 8 for 9 h and line no. 13 , 14 for 24 h), 5-FU-AM hydrogel treated rats (line no. 3 and 4 for 3 h, line no. 9, 10 for 9 h and line no. 15 , 16 for 24 h) and 5-FU/AM-MMT composite hydrogel treated rats (line no. 5 , 6 for 3 h, line no. 11 , 12 for 9 h and line no. 17, 18 for 24 h), line no 19 , 20 was indicted control group without any treatments

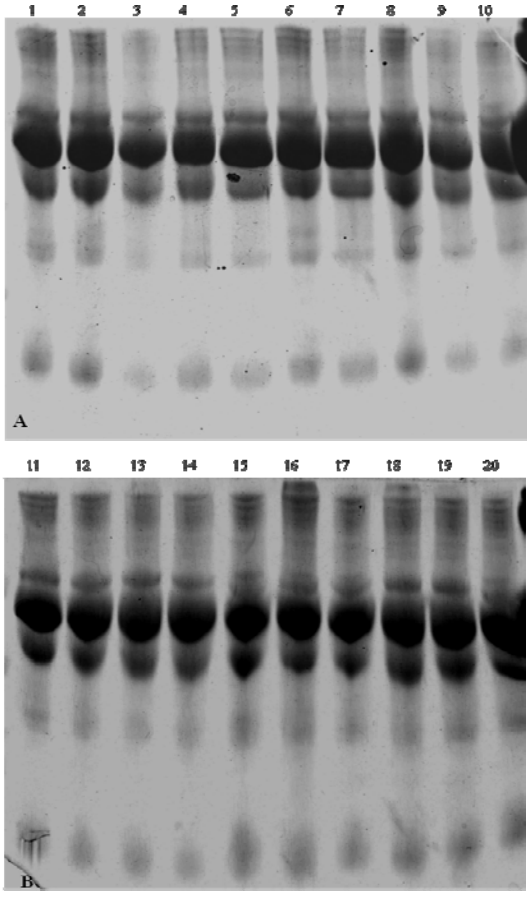

Fig. S10

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